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We have studied various aspects of HIV biology within the monocyte-macrophage. These studies included interactions of newly discovered cytokines in promoting monocytic cell development in the context of HIV infection. Both interleukin-3 and kit ligand/stem cell factor were found to augment myelopoiesis in vitro and protect monocyte-macrophage from toxic effects of Zidovudine and ganciclovir. Kit ligand/stem cell factor had no upregulatory effect on HIV transcription while interleukin-3 did. These studies set the stage for the use of these cytokines to reduce the cytotoxicity of important agents such as Zidovudine and ganciclovir in the treatment of HIV disease and its complicating opportunistic infections. We also discovered that the long-terminal repeat (LTR) of HIV contains motifs responsive to steroid hormone receptors. There appears to be a complex interplay among these transcription factors in terms of modulating virus expression in the monocyte-macrophages. These studies open a new avenue of research with regard to therapeutics based on retinoic acids. Efforts were initiated to introduce synthetic genes capable of inhibiting HIV into the monocyte-macrophages in novel adenoassociated virus (AAV) vectors. We have successfully constructed prototype vectors using the AAV backbone and found a high transduction efficiency in macrophages. Future studies will examine the optimal constructs to inhibit HIV replication in these cells

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FOREWORD

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Final Report
(Period 09/28/90 - 09/27/93)
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Pathobiology of HIV in the Human Monocyte-Macrophage

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INTRODUCTION

Our research program explores the interactions of HIV with monocyte-macrophages. Monocyte-macrophages are important host defense cells which both interact with invading pathogens and present antigens to other immune effector cells. HIV is able to infect monocyte-macrophages via the CD4 cell surface structure and both replicate within these cells as well as potentially alter their function (1-3). The purpose of the present work is to determine the regulation of HIV within this cell type, the consequences of infection particularly with regard to cytokine generation, and to ultimately use this information for the clinical benefit of individuals with HIV disease.

BODY

A. Cytokine Biology

We pursued the identification of a novel cytokine termed "kit ligand/stem cell factor" which is produced by mesenchymal cells including bone marrow stromal fibroblasts (4,5). Because this cytokine was considered as a potential therapeutic in stimulating stem cell proliferation for purposes of immune reconstitution, we addressed whether the kit ligand/stem cell factor might alter HIV replication in monocyte-macrophages. Standard cultures of monocyte-macrophages were established in vitro using tropic isolates of HIV including BAL and 9533. We found no upregulation or effect on HIV within the monocyte-macrophage following exposure of the cells to kit ligand/stem cell factor.

This suggested to us that this cytokine, as well as other early acting hematopoietic growth factors, might of therapeutic use in people with HIV. To that end, we studied myeloid progenitor development (CFU-GM) and erythroid progenitor development (BFU-E) in vitro in the presence of important therapeutics for people with AIDS including AZT, ganciclovir as well as the inflammatory cytokine tumor necrosis factor alpha (TNF-α) (Table 1) (Figure 1).

We had previously determined, as had other groups, that TNF-α could upregulate HIV expression in monocyte-macrophages as well as T cells (6,7). Comparative in vitro studies in the laboratory examined the effects of recombinant human interleukin-3 with those effects from recombinant kit ligand/stem cell factor. We found that there were protective effects against both myelotoxic drugs and inflammatory cytokines such as TNF-α utilizing interleukin-3, with a discernable but less potent protective effect seen with kit ligand/stem cell factor. We confirmed that interleukin-3 may moderately upregulate HIV replication in monocyte-macrophages. Using these data, we established a framework upon which certain cytokines such as the kit ligand/stem cell factor may be used to promote the development of myeloid cells, including monocyte-macrophages, in the presence of HIV infection without stimulating replication of the retrovirus.

B. Cytokine Signalling Pathways in Monocyte-Macrophages

Based on our prior studies of TNF-α effects on HIV in monocyte-macrophages, we sought to further explore the pathways of signal transduction in myeloid cells in the context of this inflammatory cytokine. The purposes of these studies was to better understand those biochemical mediators in the monocyte-macrophage which could contribute to upregulation of HIV expression and thereby to seek novel antiviral interventions. We focused on the sphingomyelin pathway, specifically the intermediate ceramide (8). We found that ceramide was a potent upregulator of HIV replication, particularly using monocytic cell lines such as U1 (a subclone of U937) in which viral transcription is minimal. We found that exogenous ceramide was a potent upregulator of HIV expression in monocytic cells, providing direct evidence for signal transduction intermediates in the TNF-sphingomyelin pathway. Potential inhibitors of this pathway might be considered as ultimate therapeutic agents for HIV disease (Figure 2 & 3).

C. Steroid Hormone Receptors

A major part of our program has involved the study of transcriptional regulation via the negative regulatory element

(NRE) of the HIV long-terminal repeat (LTR). The NRE has motifs which could interact with the family of transcription factors that act as steroid hormone receptors. We first found that a newly identified steroid hormone receptor termed ARP tightly bound to a motif in the NRE. We also found that there was a complex interaction among the many members of this steroid hormone receptor family with regard to homodimer and heterodimer formation. Most potent with regard to binding to the responsive motifs in the HIV negative regulatory element were the steroid hormone receptors for trans-retinoic acid and 9-cic-retinoic acid (RAR-α and RXR-α) (9) (Figure 4).

We recently found that there was an excellent correlation with regard to downregulation of HIV expression in different monocyte-macrophage cell lines and in different T cell lines with regard to level of expression of these steroid hormone receptors in the cells. This correlation provides indirect but important data that retinoids may be useful in modulating HIV replication in different cell types (Figure 5).

D. Transcription Regulation of HIV, Independent of the NF-Kappa B Structure

We found that certain cytokines could cause transcriptional activation by the HIV LTR independent of the NF-kappa B structure. This was most dramatically seen in viruses with deletion point mutations in the two NF-kappa B sequences present in the HIV LTR. A primitive cell line with myeloid and megakaryocytic properties called CMK was studied in this regard. Induction of differentiation with the phorbol diester PMA led to transactivation despite the absence of intact NF-kappa B sequences. In the context of the pathobiology of HIV in the monocyte-macrophage, we then focused on GM-CSF treatment of THP-1 monocytic cells transfected with these NF-kappa B mutants. There was excellent transactivation of these cells as well. Sequential deletions in the LTR demonstrated the minimal sequences required for transactivation which are clustered around the TATA box of the HIV LTR.

E. Transduction of Monocyte-Macrophages with Adenoassociated Virus Vectors (AAV)

In order to dissect the effects of different gene products within the monocyte-macrophage, as well as use artificial genes which might inhibit HIV, we sought to identify a vector system which had a high transduction efficiency for resting cells such as macrophages. We have been fortunate in obtaining adenoassociated virus (AAV) vector and have conducted a series of studies to determine ability of these vectors to transduce monocyte-macrophages. Our initial studies are very promising. Using specific marker genes including CAT as well as Beta-Gal, we have found excellent transduction efficiency (as high as 50%) in some experiments. No cytopathic effects of the AAV vector have been discernable in the monocyte-macrophage.

We will now proceed by introducing specific HIV gene products, including VPR and VPU, into the monocyte-macrophage under the control of various promoters. The effects of the gene products on monocyte-macrophage function with regard to antigen presentation in cytokine generation will be studied. In addition, we will introduce antisense constructs for purposes of inhibiting HIV within the monocyte-macrophage by exploiting the AAV vector.

CONCLUSION

The program has succeeded in addressing many of the goals set out three years ago. We have an improved understanding of signal transduction pathways of important cytokines such as TNF-α within the monocyte-macrophage. We have identified the novel cytokine kit ligand/stem cell factor which does not upregulate HIV within the monocyte-macrophage but leads to enhanced progenitor development, thereby providing an opportunity for potential stem cell expansion and immune reconstitution. A new mechanism of transcriptional activation of HIV in the monocyte-macrophage via steroid hormone receptor interaction with motifs in the HIV LTR was identified. Manipulation of different retinoids and other steroids could lead to development of novel therapeutics. Finally, the first steps in

gene therapy targeting resting cells such as macrophages were taken with a new class of vectors, AAV, with very positive results to date.

The work supported by this contract thereby contributed to both the basic understanding of the pathobiology of HIV in the monocyte-macrophage as well as potential new therapeutic interventions for people with HIV disease.

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APPENDIX

Table 1

Figure 1 through 5

FIGURE LEGEND

Figure 1: BFU-E (left column) or CFU-GM (right column) in the presence of ZDV (0, 0.05, 0.1 and 1.0 μM), GAN (0, 0.1, 0.2, and 1.0 μM), IFN (0, 100, 1000 and 5000 U/ml), TGF (0, 0.04, 0.4, 2.0 ng/ml) or TNF (0, 0.4, 4.0 and 20 ng/ml) and control, SCF (10 ng/ml) or IL-3 (10 ng/ml). Data presented are the mean and SE.

Figure 2: Time course of HIV expression by ceramide treated cells.

Panel A: HIV-1 core p24 production by U-1_{IIIB} cells. ●: control, ■: ceramide treated cells.

Panel B: HIV-1 core p24 production by OM-10.1 cells treated with 50 μM ceramide. ●: control, ■ : ceramide treated cells.

Panel C: Relative cell growth in the absence or presence of 50 μM ceramide. ▲ and Δ: OM-10.1; ■ and □: U-1_{IIIB} cells. Open symbols represent controls, full symbols represent ceramide treated cells. Initial cell density: 5 x 10^5 cells/ml.

Panel D: Cell viability in the absence (open symbols) or presence of 50 μM ceramide.

Figure 3: Enhancement of CAT transcription driven by the HIV LTR by ceramide. U-937 cells transfected by the pU3R-III CAT/h plasmid were exposed to increasing concentrations of ceramide, DMSO vehicle or PMA as a positive control and CAT activities were determined at day 5. Ceramide induced a dose dependent increase in CAT activity.

Figure 4: Interaction of the nuclear receptors ARP-1, EAR-3, EAR-2, HNF-4, NGFI-B, and RXR-α with sequences located within the HIV-1 LTR.

Panel A: Schematic representation of the HIV-1 LTR. Nucleotide numbers are relative to the transcription start site (+1). The double-stranded oligo NRRE-1 spanning the -320 to -358 LTR region is bracketed. Asterisks and open circles indicate nucleotides whose methylation interfered strongly and weakly, respectively, with receptor binding. The coding strand of the double-stranded oligo NMUT-BD harboring eight nucleotide substitutions (white letters) is also shown. NRRE-1 and NMUT-BD oligos have a 5′ GATC overhang.

Panel B: DNase I protection of the -453 to -244 DNA region of the HIV_{LAI} LTR ^{32}P-labeled at the coding or noncoding strands in the presence of COS-1-produced receptors, as indicated. Lanes G+A: chemical sequencing ladder; control: DNase I protection using whole-cell extracts from mock-transfected COS-1 cells. Solid bars indicate areas protected by ARP-1, EAR-3, and EAR-2; open bars indicate areas protected by HNF-4.

Panel C: Methylation interference analysis of in vitro translated ARP-1, EAR-3, EAR-2, and HNF-4 with an oligo probe spanning the -368 to -310 region of the HIV_{LAI} LTR ^{32}P-labeled at the coding or noncoding strands, as indicated. Lanes M: G+A sequencing ladder; B: protein-bound probe; and F: free probe. Methylation interference symbols as in A.

Panel D: Electrophoretic mobility shift analysis or nuclear receptor binding to the NRRE-1 probe in the presence (+) or absence (-) of 100-fold molar excess of the indicated unlabeled oligo

competitors and 9-cis retinoic acid (10^{-6} M). NS: non-specific oligo with unrelated sequence.

Figure 5: Retinoic acid effects on HIV-1 IIIB acute infection in T cell and monocytoid cell lines. On day 17 after infection, p24 antigen level and MTT dye conversion was measured. To compensate for nonspecific effects on cell viability the ratio of p24 antigen/MTT dye conversion is presented.

Panel A: H9 and CEM cells

Panel B: U937 and THP-1 cells

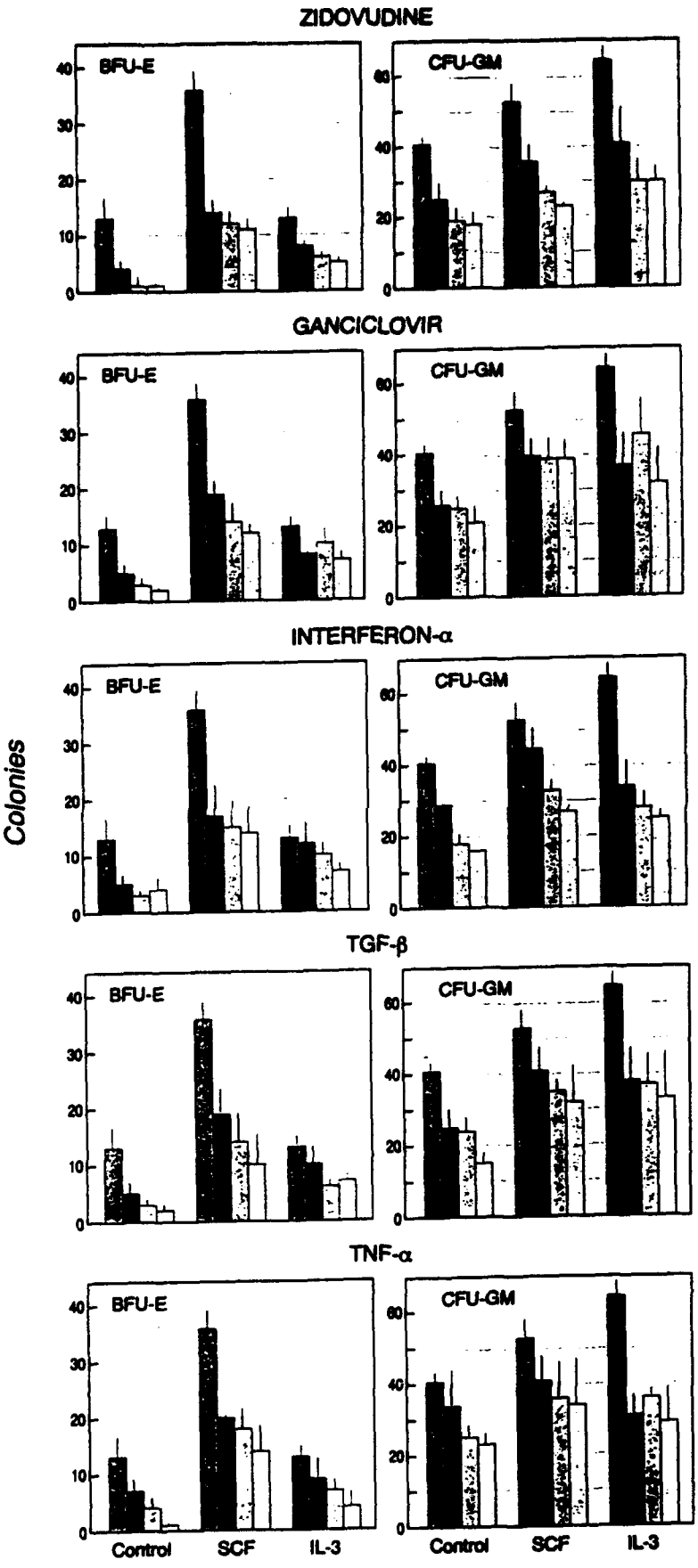
Panel C: The same experiment was performed on THP-1 cells 3 months after infection

The data presented is the mean of three experiments, each performed in duplicate.

IC50

	ZDV uM	GAN uM	IFN U/ml	TNF ng/ml	TGF ng/ml
BFU CONT	0.5 +/- .1	.55 +/- .1	3500 +/- 1300	10 +/- 1	1.3 +/- .3
BFU SCF	0.8 +/- .2	.8 +/- .1	5000 +/- 500	18 +/- 10	1.3 +/- .8
BFU IL3	*1.0 +/- .2	.9 +/- .6	6000 +/- 1000	16 +/- 3	2.4 +/- .3
GM CONT	1.2 +/- .05	1.2 +/- .6	4000 +/- 400	25 +/- 13	1.5 +/- .4
GM SCF	1.1 +/- .1	1.7 +/- .3	5600 +/- 400	40 +/- 8	2.5 +/- 1.5
GM IL3	1.2 +/- .1	1.2 +/- .4	5200 +/- 400	25 +/- 5	2.5 +/- 1.3

(Table 1)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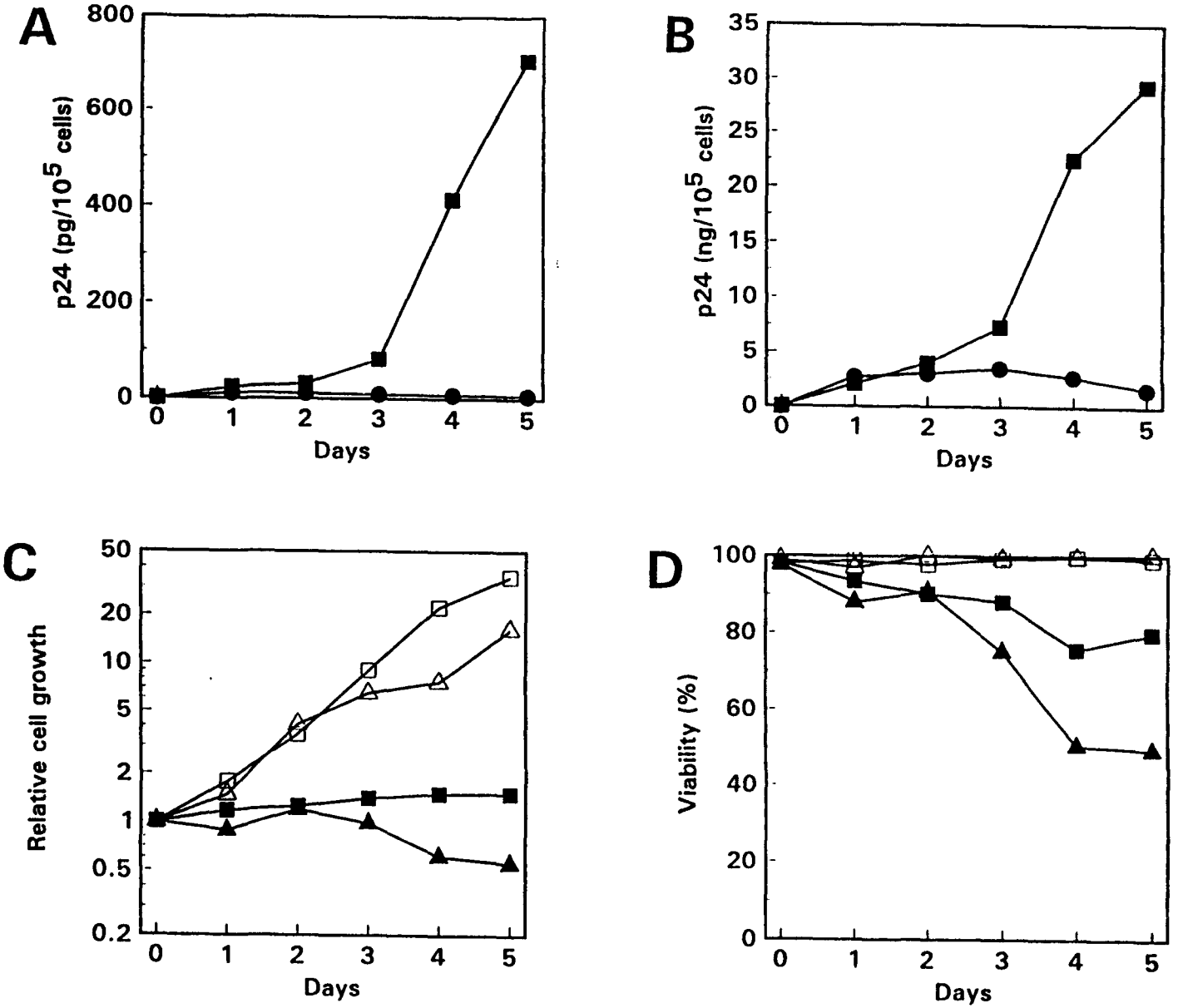
Control
SCF
IL-3
Control
SCF
IL-3

(Figure 1)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p24 (ng/10^5 cells)
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Viability (%)
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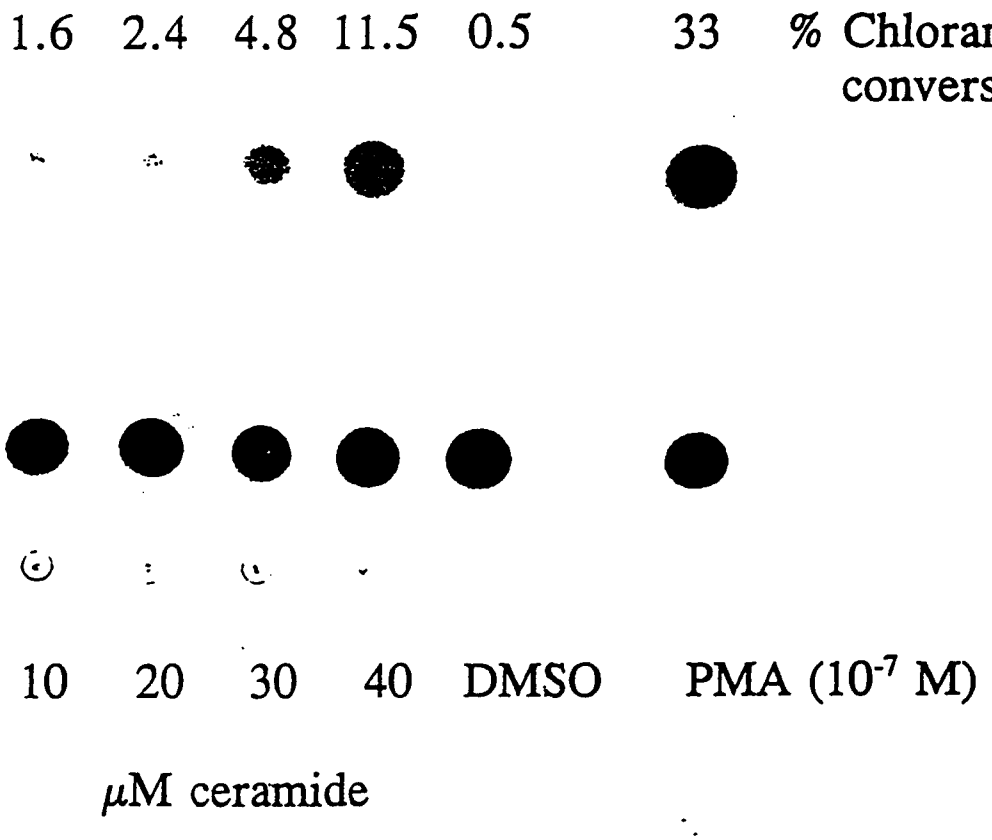
Days

(Figure 2)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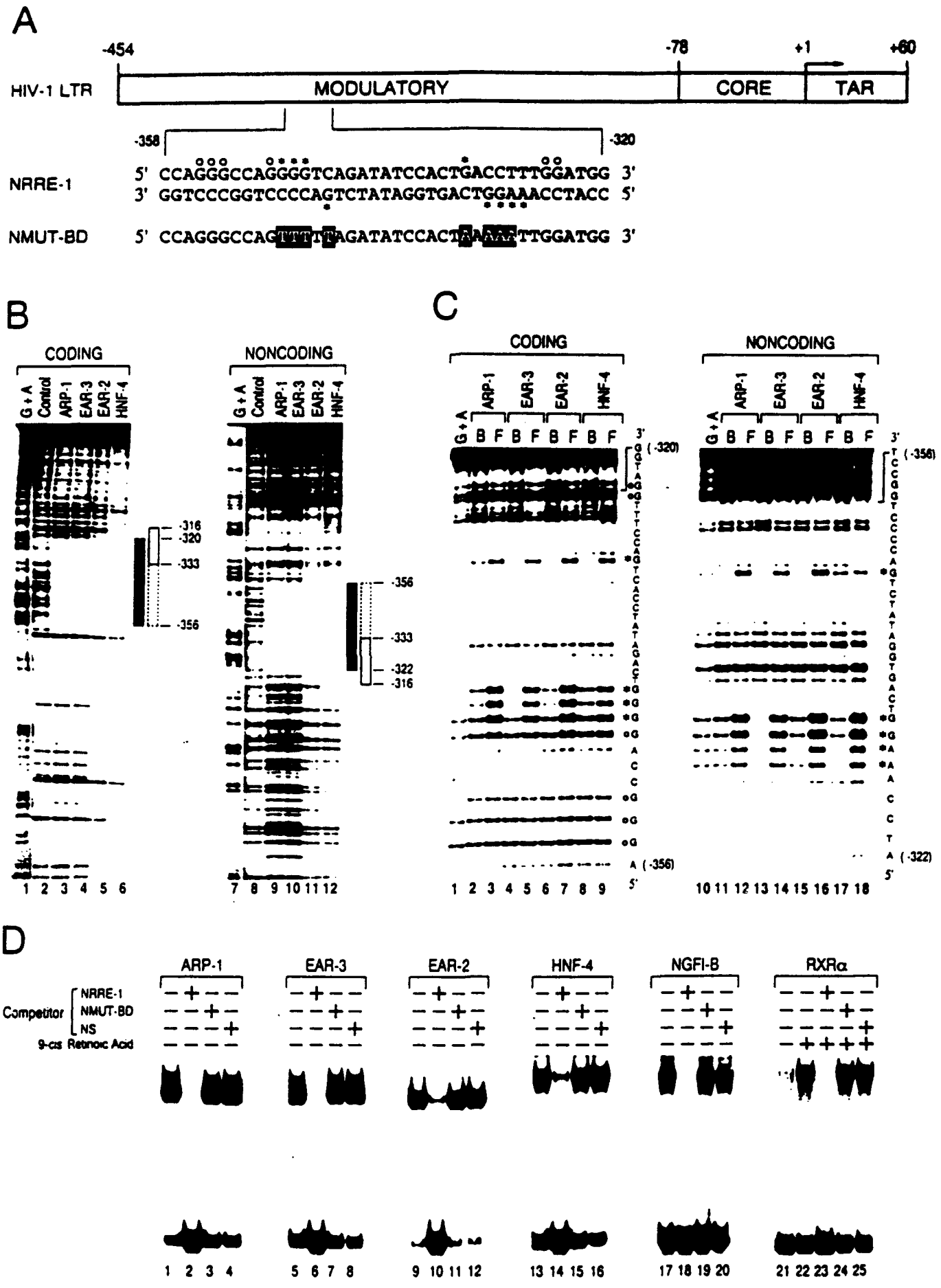
10 20 30 40 DMSO PMA (10^{-7} M)
μM ceramide

(Figure 3)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TAR
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NRRE-1
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3' GGTCCCGGTCCCCAGTCTATAGGTGACTGGAAACCTACC 5'
NMUT-BD
5' CCAGGGCCAGTTTTTAGATATCCACTAAAATTGGATGG 3'
B
CODING
NONCODING
G+A
Control
ARP-1
EAR-3
EAR-2
HNF-4
-316
-320
-333
-356
-322
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7 8 9 10 11 12
C
CODING
NONCODING
ARP-1
EAR-3
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HNF-4
G+A
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(-356)
(-322)
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ARP-1
EAR-3
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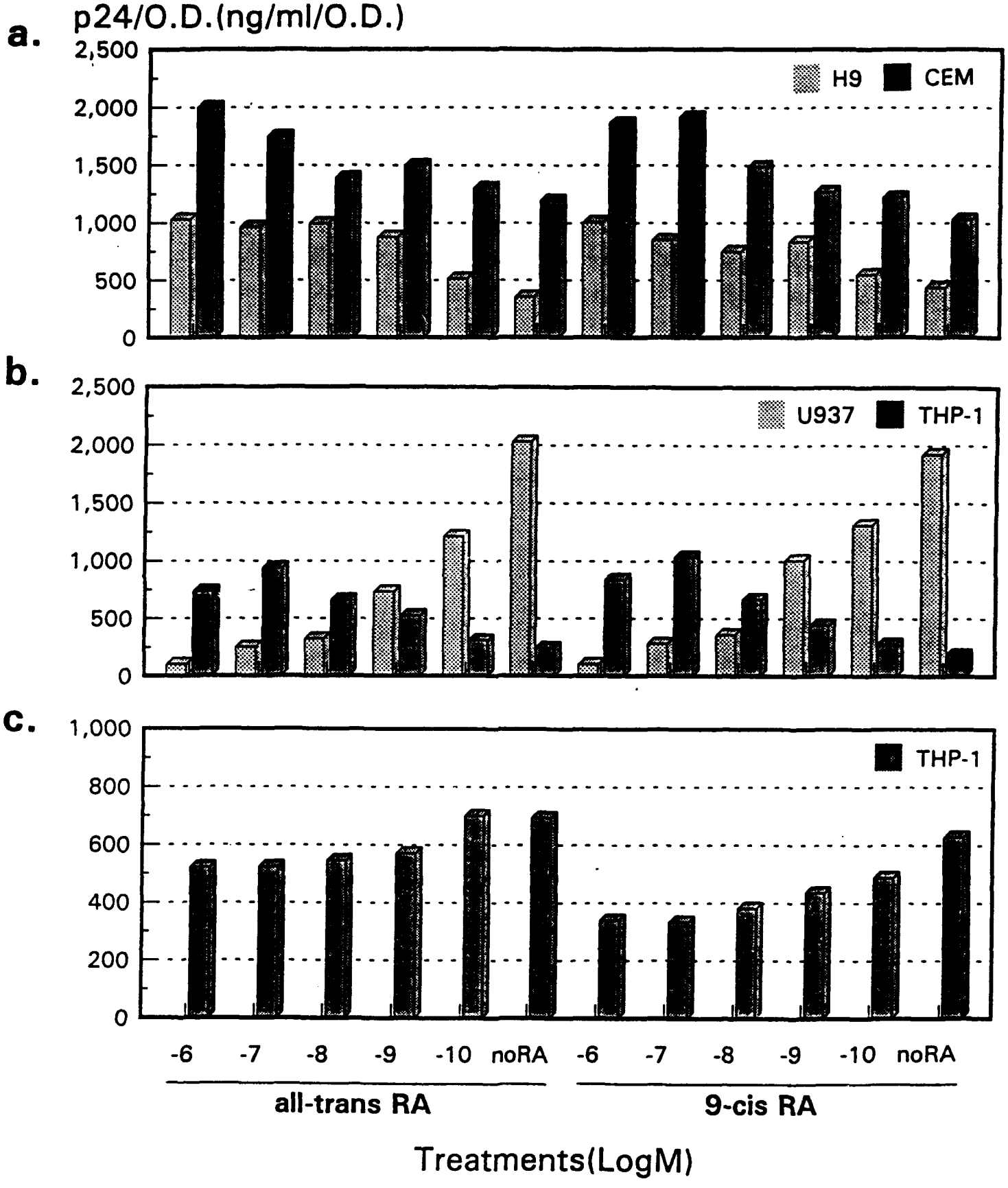
(Figure 4)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noRA
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Treatments(LogM)

(Figure 5)